Endogenous Controls for Real-Time Quantitation of miRNA Using TaqMan® MicroRNA Assays.

Introduction

MicroRNAs (miRNAs) are small non-coding RNAs whose function has been implicated in a wide range of fundamental cellular processes including cell proliferation, cell differentiation, and cell death. Quantitation of miRNA gene expression levels has become an essential step in understanding these mechanisms, and has shown great promise in identifying effective biomarkers correlative with human disease[1,2]. Applied Biosystems has developed an extensive set of TaqMan® MicroRNA Assays, novel stem-loop RT and real-time PCR assays, for the quantitation of mature miRNA expression[3]. TaqMan® Assays are the ideal choice for these applications because of their unsurpassed sensitivity, specificity, and wide dynamic range. Additionally, far less input material is required compared to microarrays and other alternative technologies.

When performing these experiments, variation in the amount of starting material, sample collection, RNA preparation and quality, and reverse transcription (RT) efficiency can contribute to quantification errors. Normalization to endogenous control genes is currently the most accurate method to correct for potential RNA input or RT efficiency biases.

Careful selection of an appropriate control or set of controls is extremely important as significant variation has been observed between samples, even for the most commonly used housekeeping genes, including ACTB (ß-Actin) and GAPDH[4]. An ideal endogenous control generally demonstrates gene expression that is relatively constant and highly abundant across tissues and cell types. However, one must still validate the chosen endogenous control or set of controls for the target cell, tissue, or treatment[5], as no single control can serve as a universal endogenous control for all experimental conditions.

When considering endogenous controls suitable for use with TaqMan MicroRNA Assays, it is important that they share similar properties, such as RNA stability and size, and are amenable to the miRNA assay design. A number of reports indicate that other classes of small non-coding RNAs (ncRNAs) are expressed both abundantly and stably, making them good endogenous control candidates. We have performed a systematic study of a set of human ncRNA species ranging in size from 45

to 200 nucleotides, including transfer RNA (tRNA), small nuclear RNA (snRNA) and small nucleolar RNA (snoRNA)[6] across a relatively wide variety of tissues and cell lines to determine their suitability as endogenous controls when quantitating miRNA expression levels using real-time PCR methods.

Materials and Methods

Small Non-coding RNAs (ncRNA)

Candidate small non-coding RNA sequences were obtained from the NCBI's GenBank database.

TaqMan® MicroRNA Assays

TaqMan MicroRNA Assays, consisting of an RNA-specific stem-looped RT primer and TaqMan® Assay (forward and reverse primers and FAM™ dye-labeled MGB probe), were designed for each candidate endogenous control RNA using Applied Biosystems miRNA-specific assay design parameters. A full list of TaqMan MicroRNA Assays is available at **mirna.appliedbiosystems.com**

TaqMan® Gene Expression Assays

The TaqMan® Gene Expression Assays for detection of endogenous control genes used in this experiment were: 18S (Hs99999901_s1), GAPDH (Hs99999905_m1), and ACTB (Hs99999903_m1). A full list of TaqMan Gene Expression Assays is available at **www.allgenes.com**

RNA Samples

Total RNA for human and mouse tissues were acquired from Ambion, Inc., an Applied Biosystems Business, and BD Biosciences. Total RNA for NCI-60 cell lines was obtained from Dartmouth Medical School.

Reverse Transcription (RT) Reaction

The RT reaction was performed using the TaqMan® MicroRNA Reverse Transcription Kit (PN 4366596, 200 reactions; or PN 4366597, 1,000 reactions). Next, 2 ng/µL of RNA, 1X stem-loop RT primer, 3.33 U/µL reverse transcriptase, 0.25 U/µL RNase inhibitor, 0.25 mM dNTPs, and 1X reaction buffer were run in a total reaction volume of 15 µL and incubated at 16°C for 30 min, and 42°C for 30 min, and 85°C for 5 min in a thermalcycler.

PCR Reaction

Following the RT step, 0.8 µL of the RT reaction was combined with 0.5 µL of a TaqMan MicroRNA Assay (20X; forward primer, reverse primer, and probe) and 5 µL of TaqMan® Universal PCR Master Mix, No AmpErase® UNG (PN 4324018) in a 10-µL final volume. Real-time PCR was performed using an Applied Biosystems 7900HT Fast Real-Time PCR System with cycling conditions of 95°C for 10 min [followed by 95°C for 15 sec and 60°C for 60 sec] for a total of 40 cycles. Each TaqMan Assay was run in quadruplicate.

Results and Discussion

TaqMan MicroRNA Assays were designed and synthesized for a total of 38 human snRNA and snoRNA (sn/snoRNA) and tRNA genes. The standard TaqMan MicroRNA Assays Protocol (PN 4364031) was followed to examine performance of the sn/snoRNA endogenous control candidate assays using 30, 3, and 0.3 ng of human lung total RNA. For each assay, a paired no-template control (NTC) reaction was performed. A subset of 23 sn/snoRNA endogenous control candidates that demonstrated good assay linearity (R^2 >0.96) and abundance (data not shown), and had NTC C_T value >38, was identified and used to screen a panel of 38 normal human tissues (Table 2, see appendix) and 59 NCI-60 cell lines (Table 3, see appendix).

These control candidates showed expression levels that remained relatively constant across the tissues and cell lines tested. A final group of ten small RNAs (Table 1), including both snRNAs and snoRNAs, was selected as the best performing control assays, based upon their expression level and stability, as determined by statistical analysis. These ten sn/snoRNA endogenous control genes demonstrated expression levels that were both relatively abundant (C_T

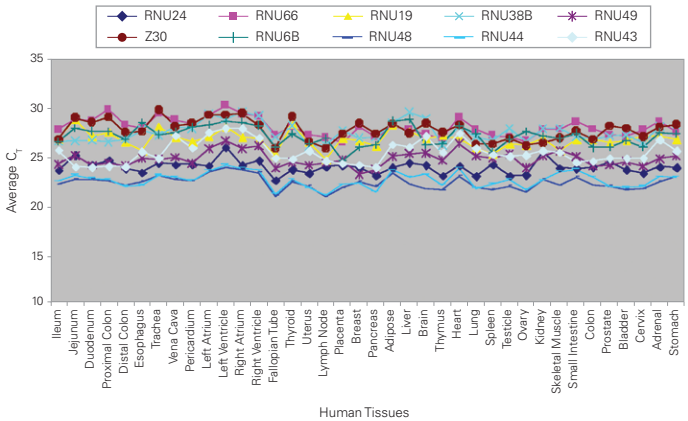

Figure 1a. Expression profile of ten human sn/snoRNAs for miRNA endogenous controls across 38 normal human tissues.

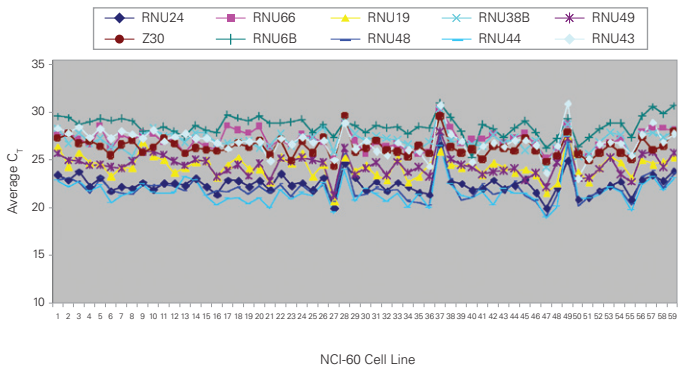

Figure 1b. Expression profile of ten human sn/snoRNAs for miRNA endogenous controls across 59 NCI-60 cell lines.

range 22–28.9) and constant across all 38 human tissues (Figure 1a) or 59 NCI-60 cell lines (Figure 1b) tested, with standard deviations for the average C_T values ranging from 0.7 to 1.1 across tissues (Table 4, see appendix), and 1.0 to 1.4 across cell lines (Table 5, see appendix).

In addition to these ten human controls, we identified an additional eight controls (Table 1). They also displayed good linearity (R^2>0.96), good abundance (average C_T range 22–28) and NTC C_T >38. These controls were also tested across the 38 tissues (Figure 2). The average C_T and StDev of the average C_T for these controls are shown in Table 4.

To compare the use of various normalization control classes, the expression patterns for sn/snoRNA endogenous controls described above were compared to miRNA control candidates and the normalization controls typically used for conventional TaqMan Assays. To identify miRNA control candidates, the expression levels of 247 miRNAs were examined using TaqMan MicroRNA Assays across all 38 normal human tissues and 59 NCI-60 cell lines (data not shown). The results showed constant expression for a subset of these miRNAs in tissues (hsa-miR-26b, hsa-miR-92, hsa-miR-92N) and cell lines (hsa-miR-423, hsa-miR-374, hsa-miR-16), indicating that within this group there may be good endogenous control candidates (Figure 4a and 4b; and Table 6, see appendix). The use of the most stably expressed miRNA(s) for a specific experimental condition to normalize miRNA expression data is a commonly used approach and poses an alternative to using the sn/snoRNA endogenous controls previously described.

For comparison, the expression patterns of the sn/snoRNA and miRNA control candidates and traditional TaqMan Assay controls were plotted together. The C_T values for the three most stable assays for sn/snoRNA and miRNA were averaged and compared to 18S rRNA, GAPDH and ACTB. The 18S rRNA, sn/snoRNA and miRNA exhibit similar patterns and show relatively constant expression across all tissues (Figure 5a) and cell lines (Figure 5b), although 18S rRNA is expressed at a significantly higher level.

Of the 18 human controls identified, RNU48, RNU44, U18, and U47 are the most highly abundant across the tissues based on average C_T. In addition to RNU44 and RNU48, the following: RNU24, RNU49, U75, and U47 display the least variability across the tissue samples, with StDev of the average C_T between 0.7 and 0.8. RNU48 and RNU44 also showed the highest abundance across the NCI-60 cell lines, although they showed highest variability compared to the other controls tested. Thus, if you were comparing miRNA expression across the 38 tissue samples, RNU48 or RNU44 would be considered good candidates for endogenous controls because they show good abundance and relatively stable expression. However, if your study were across the NCI-60 cell line, you may consider one of the other controls such as RNU6B, Z30, or RNU38B, as RNU44 and RNU48 display greater variability.

Mouse Endogenous Controls

A similar approach was taken to identify mouse-specific sn/snoRNA endogenous controls. As with the human selections, a number of candidate mouse snoRNAs were tested across various tissues, and those showing relatively high abundance and the least variation were chosen. These experiments identified five mouse snoRNAs (Table 1) that fit the same criteria set for the human endogenous controls. Figure 3 shows the expression profile across 12 tissues for these five mouse snoRNAs,

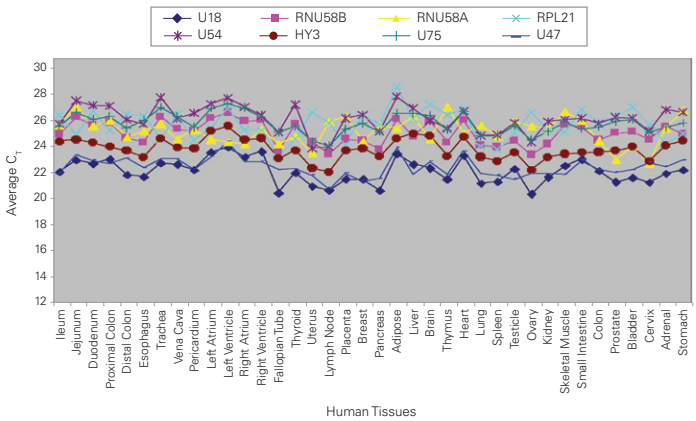

Figure 2. Expression profile of eight additional human sn/snoRNAs for miRNA endogenous controls across 38 normal human tissues.

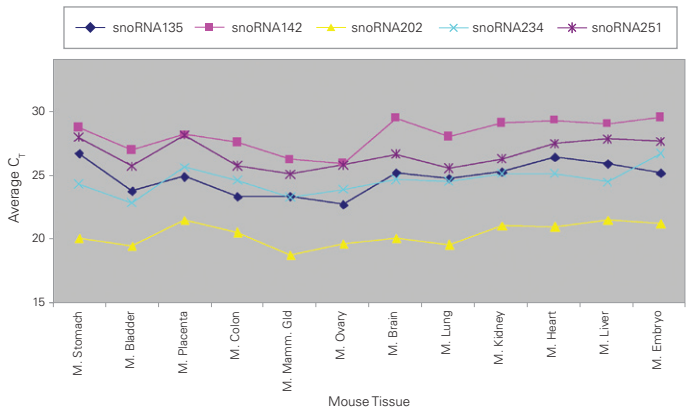

Figure 3. Expression profile of five mouse sn/snoRNAs for miRNA normalization across 12 normal tissues.

including the average C_T, and Table 7 (see appendix) shows StDev of the average C_T. Mouse snoRNA202 showed the highest abundance and least variability across the 12 tissues, making it the best candidate if your study were to examine miRNA expression across these 12 tissues.

Recommendations for miRNA Expression Data Analysis

This study confirms that both 18S rRNA and a group of 23 small ncRNAs not related to miRNAs are invariantly expressed across a relatively broad tissue panel. Additionally, we demonstrate that specific miRNA species that are similarly invariantly expressed across the same panel of samples can be identified. These results suggest that effective data normalization can be achieved using a variety of endogenous controls; however, there are significant advantages to using the sn/snoRNA endogenous controls for normalizing TaqMan MicroRNA Assay expression data:

- snRNA and snoRNA genes are closer in size (length) to miRNAs (<200 bp)
- snRNAs and snoRNAs are constitutively and abundantly expressed across a large number of tissues and cell lines
- snRNA, snoRNA, and miRNA assays were designed using identical approaches
- snRNA and snoRNAs are unlikely to be involved in miRNA regulatory pathways

Applied Biosystems offers the 18 human snRNA and snoRNA and five mouse snoRNA endogenous control assays (Table 1) that are recommended for normalizing human and mouse TaqMan MicroRNA Assays. Regardless of the gene or gene set that is chosen, we highly recommend that the consistency of expression be reconfirmed under the specific conditions of the experiment.

The following steps are recommended when selecting endogenous controls for miRNA data normalization:

1. Carefully select a set of several endogenous control genes based on the species, tissues, or cell lines used in your study. This will often require screening available TaqMan MicroRNA Assays for those sn/snoRNA endogenous controls that perform best in the specific samples under investigation. Alternatively, or in addition to, use specifc miRNAs that demonstrate the least variability across experimental conditions under consideration. From the previously described study, based upon data generated across a wide variety of tissues and cell lines, we have identified the following candidate control genes as showing the least variability:
   - sn/snoRNAs:
     - Human: RNU48, RNU44, U47, RNU6B, or all four
     - Mouse: snoRNA202, snoRNA234, or both
   - miRNAs:
     - Tissues: hsa-miR-26b, hsa-miR-92, hsa-miR-92N
     - Cell lines: hsa-miR-423, hsa-miR-374, hsa-miR-16
   - Traditional TaqMan Assay controls: 18S rRNAs
2. Normalize your C_T values using the average C_T of the endogenous controls
3. Use ΔC_T (miRNA C_T – averaged endogenous control C_T) or fold-change relative to a calibrator or reference sample ($2^{-\Delta\Delta C_T}$) for relative expression analysis

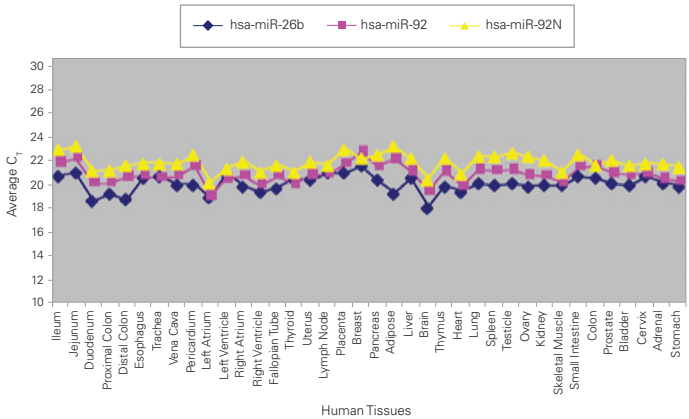

Figure 4a. Expression profile of three human miRNA candidates for miRNA normalization across 38 normal human tissues.

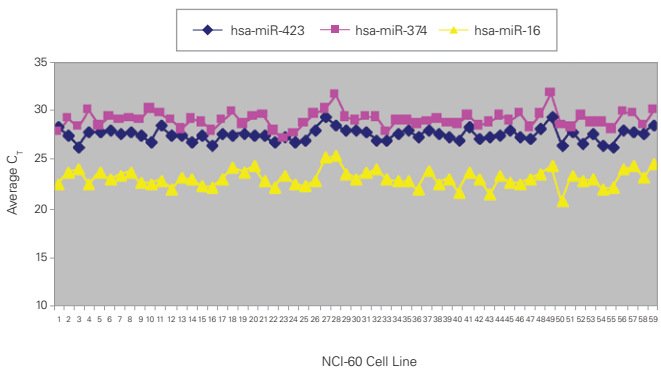

Figure 4b. Expression profile of three human miRNA candidates for miRNA normalization across 59 NCI-60 cell lines.

Conclusion

We have identified a set of 18 endogenous control candidates in human, and five in mouse, that can be used to normalize miRNA gene expression data. In this study, we found the following six sn/snoRNA endogenous controls show the highest abundance and least variability across normal tissues and cell lines that were tested: Human: RNU48, RUN44, U47, and RNU6B; Mouse: snoRNA202 and snoRNA234. Stable expression patterns across tissues and cell lines were also observed for specific miRNA control candidates and 18S rRNA, providing additional normalization options.

Authors

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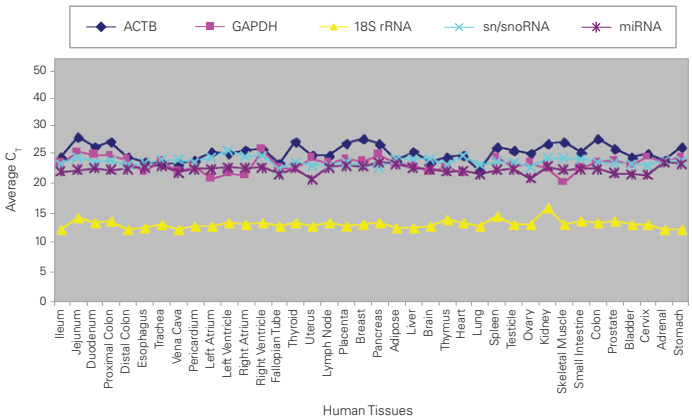

Figure 5a. Comparison of the expression pattern for different types of normalization controls across 38 normal human tissues: sn/snoRNA, miRNAs, and TaqMan® Assay controls.

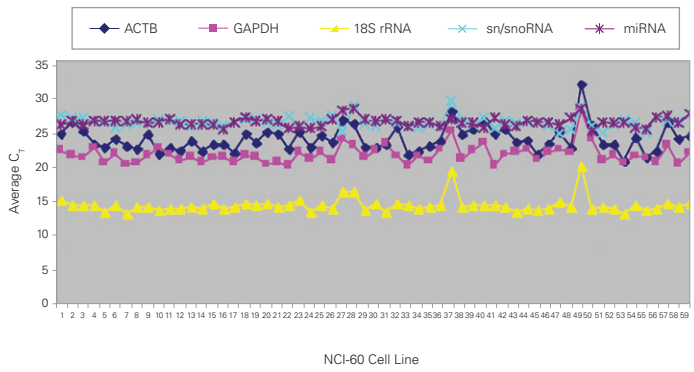

Figure 5b. Comparison of the expression pattern for various types of normalization controls across 59 NCI-60 cell lines: sn/snoRNA, miRNAs, and TaqMan® Assay controls.

Appendix

TABLE 1. List of human and mouse miRNA endogenous controls (sn/snoRNAs). Human sn/snoRNAs, highlighted in dark green, were tested with tissue and cell lines. Human sn/snoRNAs, highlighted in light green, were tested with tissues only. Mouse sn/snoRNAs, highlighted in blue, were tested only with tissues.

Human Gene (NCBI Symbol)	Gene Name	NCBI Accession	Alias	Product Name	AB Assay ID	Part Number	Tissue	NCI-60
SNORD24	small nucleolar RNA, C/D box 24	Z48765	U24; RNU24	RNU24	001001	4373379	Yes	Yes
SNORA66	small nucleolar RNA, H/ACA box 66	NR_002444	U66; RNU66	RNU66	001002	4373382	Yes	Yes
SNORA74A	small nucleolar RNA, H/ACA box 74A	X94290	U19; RNU19	RNU19	001003	4373378	Yes	Yes
SNORD38B	small nucleolar RNA, C/D box 38B	NR_001457	U38B; RNU38B	RNU38B	001004	4373380	Yes	Yes
SNORD49A	small nucleolar RNA, C/D box 49A	NR_002744	U49; U49A; RNU49	RNU49	001005	4373376	Yes	Yes
SNORD48	small nucleolar RNA, C/DR box 48	NR_002745	U48; RNU48	RNU48	001006	4373383	Yes	Yes
SNORD7	small nucleolar RNA, C/D box 7	AJ007733	Zn0; mgU6-47	Z30	001092	4373377	Yes	Yes
RNU6B	U6B, small nuclear	NR_002752	U6	RNU6B	001093	4373381	Yes	Yes
SNORD44	small nucleolar RNA, C/D box 44	NR_002750	U44; RNU44	RNU44	001094	4373384	Yes	Yes
SNORD43	small nucleolar RNA, C/D box 43	NR_002439	U43; RNU43	RNU43	001095	4373375	Yes	Yes
NA*	U18 snoRNA/RPL4[1]	AB061820[3]	U18	U18	001204	4380904	Yes	No
SNORD58B	small nucleolar RNA, C/D box 58B	NR_002572	U58b; RNU58B	RNU58B	001206	4380905	Yes	No
SNORD58A	small nucleolar RNA, C/D box 58A	NR_002571	U58a; RNU58A	RNU58A	001207	4380906	Yes	No
NA*	RPL21/snoRNA[2]	AB061826[3]	NA	RPL21	001209	4380907	Yes	No
SNORD54	small nucleolar RNA, C/D box 54	NR_002437	U54; RU54	U54	001210	4380908	Yes	No
RNY3	Y3 small cytoplasmic (associated with Ro protein)	AC005251[3]	HY3; Y3	HY3	001214	4380909	Yes	No
SNORD75	small nucleolar RNA, C/D box 75	AF141346	U75	U75	001219	4380910	Yes	No
SNORD47	small nucleolar RNA, C/D box 47	AF141346	U47; RNU47	U47	001223	4380911	Yes	No
Mouse Gene	**Gene Name**	**NCBI Accession**	**Alias**	**Product Name**	**AB Assay ID**	**Part Number**	**Tissue**	**NCI-60**
snoRNA135	clone MBII-135 C/D box snoRNA	AF357323	snoRNA135	snoRNA135	001230	4380912	Yes	No
snoRNA142	clone MBII-142 C/D box snoRNA	AF357324	snoRNA142	snoRNA142	001231	4380913	Yes	No
snoRNA202	clone MBII-202 C/D box snoRNA	AF357327	snoRNA202	snoRNA202	001232	4380914	Yes	No
snoRNA234	clone MBII-234 C/D box snoRNA	AF357329	snoRNA234	snoRNA234	001234	4380915	Yes	No
snoRNA251	clone MBII-251 C/D box snoRNA	AF357332	snoRNA251	snoRNA251	001236	4380916	Yes	No

*No Gene Symbol or Name cited on NCBI
1. U18 snoRNA is located within RPL4 gene (intron)
2. snoRNA is located within RPL21 gene (intron)
3. DNA accession number

TABLE 2. Human and mouse tissues and part numbers.

Human Tissue	Ambion PN
Ileum	6828
Jejunum	6830
Duodenum	6832
Proximal Colon	6834
Distal Colon	6836
Esophagus	6842
Trachea	6846
Vena Cava	6848
Pericardium	6852
Left Atrium	6854
Left Ventricle	6856
Right Atrium	6858
Right Ventricle	6860
Fallopian Tube	6862
Thyroid	6872
Uterus	7892
Lymph Node	7894
Placenta	7950
Breast	6952
Pancreas	7954
Adipose	7956
Liver	7960
Brain	7962
Thymus	7964
Heart	7966
Lung	7968

Human Tissue	Ambion PN
Spleen	7970
Testicle	7972
Ovary	6974
Kidney	7976
Skeletal Muscle	7982
Small Intestine	7984
Colon	7986
Prostate	7988
Bladder	7990
Cervix	6992
Adrenal	7994
Stomach	7996
Mouse Tissue	**Ambion PN**
Ovary	7824
Brain	7812
Lung	7818
Kidney	7826
Heart	7816
Liver	7810
Embryo	7828
Mouse Tissue	**BD Biosciences PN**
Stomach	636617
Bladder	636668
Placenta	636672
Colon	636669
Mammary Gland	636670

TABLE 3. Key for NCI-60 cell line. The number corresponds to the cell line in Figures 1b, 3b, and 5.

Cell Line	Cell Line
1. SNB-19	31. MDA MB 435
2. HCT-116	32. SK-MEL-5
3. EKVX	33. NCI-H23
4. BT-549	34. HOP 92
5. A549 ATCC	35. OVCAR-8
6. TK-10	36. HS 578
7. LOXIMVI	37. SK-MEL-2
8. NCI-ADR-RES	38. COLO-205
9. SK-OV-03	39. K562
10. T-47D	40. HL-60TB
11. RXF393	41. HCC-2998
12. OVCAR-5	42. MAL-ME-3M
13. PC-3	43. CCRF-CEM
14. SF295	44. A498
15. SF539	45. SN12
16. OVCAR-3	46. MCF7
17. SK-MEL-28	47. IGROV-1
18. ACHN	48. OVCAR-4
19. CAKI-1	49. MOLT4
20. UACC-62	50. RPMI 8226
21. KM12	51. SW 620
22. U251	52. HCT-15
23. NCI-H522	53. SNB-75
24. 786-0	54. NCI-H322M
25. DU-145	55. SF268
26. NCI-H226	56. UO-31
27. NCI-H460	57. HT-29
28. SR	58. HOP-62
29. UACC 257	59. M14
30. MDA MB 231	

TABLE 4. Average C_T and StDev of the average C_T for 18 sn/snoRNAs for miRNA endogenous controls across 38 human tissues.

Across 38 Tissues

Control	Average C_T	$StDev_{CT}$
RNU24	23.9	0.7
RNU66	27.9	1.0
RNU19	26.7	0.9
RNU38B	27.5	1.0
RNU49	24.7	0.7
Z30	27.6	1.1
RNU6B	27.0	1.1
RNU48	22.2	0.8
RNU44	22.6	0.8
RNU43	25.5	1.1
U18	22.1	0.9
RNU58B	25.0	0.9
RNU58A	25.1	1.1
RPL21	25.7	1.1
U54	26.1	1.0
HY3	23.8	0.8
U75	25.7	0.8
U47	22.4	0.7

TABLE 5. Average C_T and StDev of the average C_T for ten sn/snoRNAs for miRNA endogenous controls across NCI- 60 cell lines.

Across 59 NCI-60 Cell Lines

Control	Average C_T	$StDev_{CT}$
RNU24	22.6	1.1
RNU66	27.2	1.1
RNU19	24.5	1.2
RNU38B	27.2	1
RNU49	24.6	1.2
Z30	26.5	1
RNU6B	28.7	1
RNU48	22.2	1.4
RNU44	21.8	1.4
RNU43	27.1	1.4

TABLE 6. Average C_T and StDev of the average C_T for three miRNAs across 38 human tissues and three miRNAs NCI- 60 cell lines.

Across 38 Tissues

	Average C_T	$StDev_{CT}$
hsa-miR-26b	21.6	0.7
hsa-miR-92	22.3	0.7
hsa-miR-92N	23.4	0.7

Across NCI-60 Cell Line

	Average C_T	$StDev_{CT}$
hsa-miR-423	27.6	0.7
hsa-miR-374	29.1	0.8
hsa-miR-16	23.2	0.9

TABLE 7. Expression level and variation for five mouse snoRNAs as endogenous controls in 12 tissues.

Across 12 Mouse Tissues

Control	Average C_T	$StDev_{CT}$
snoRNA135	24.7	1.3
snoRNA142	28.1	1.2
snoRNA202	20.3	0.9
snoRNA234	24.5	1.0
snoRNA251	26.6	1.1

Printed in the USA, 08/2010 Publication 127AP11-01

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